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Intramitochondrial regulation of fatty acid β-oxidation occurs between flavoprotein and ubiquinone

A role for changes in the matrix volume

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1. Rat liver mitochondria were incubated in media of different osmolarities and in the presence of various substrates. Rates of oxygen consumption and mitochondrial matrix volumes were measured in the presence and absence of ADP and uncoupler. 2. Duroquinol oxidation was insensitive to matrix volume, whereas other substrates tested showed increased rates of oxidation when the matrix volume increased from 1.0 to 1.5 μl/mg of protein; this is the range of values measured *in situ* [Quinlan, Thomas, Armston & Halestrap (1983) Biochem. J. **214**, 395–404]. 3. Palmitoylcarnitine, octanoate and butyrate oxidations were particularly sensitive to the matrix volume, increasing from negligible rates to maximal rates within this range. 4. Swelling induced by K^+ uptake also stimulated palmitoylcarnitine oxidation. 5. A similar effect of volume on substrate oxidation was seen when ferricyanide in the presence or absence of ubiquinone-1 replaced oxygen as terminal electron acceptor. 6. Measurement of flavoprotein reduction ($A_{460-480}$) demonstrated that the locus of the effect of matrix volume is between the electron-transfer flavoprotein and ubiquinone. 7. It is suggested that volume-mediated regulation of fatty acid and proline oxidation may be an important component of the hormonal stimulation of their oxidation.

INTRODUCTION

Small changes in the intramitochondrial matrix volume have the potential to regulate a variety of intramitochondrial processes (see Halestrap *et al.*, 1985*a*,*b*). We have proposed that this may be the means by which cyclic AMP-dependent and Ca^{2+}-mobilizing hormones exert their effects on mitochondrial metabolism in the intact hepatocyte (Quinlan *et al.*, 1983; Halestrap *et al.*, 1985*a*,*b*). Both classes of hormones cause an increase in the rate of fatty acid oxidation, although only glucagon is markedly ketogenic in the fed state (Sugden & Williamson, 1981; Sugden & Watts, 1983; Kosugi *et al.*, 1983). This can be attributed to an inhibition by glucagon of acetyl-CoA carboxylase, a consequent fall in tissue malonyl-CoA concentration and thus a decrease in inhibition of carnitine acyltransferase 1 (see Sugden & Williamson, 1981; McGarry & Foster, 1983). The increased activity of this enzyme stimulates the entry of fatty acids into the mitochondria and hence their oxidation. However, this is unlikely to be the only point at which hormones regulate the oxidation of fatty acids, for several reasons. The Ca^{2+}-mobilizing hormones have been reported to have both a stimulatory and an inhibitory effect on liver acetyl-CoA carboxylase under some conditions (Sugden *et al.*, 1980; Ly & Kim, 1981; Assimacopoulos-Jeannet *et al.*, 1981), but they do increase oxidation of fatty acids around the citric acid cycle (Sugden *et al.*, 1980; Sugden & Watts, 1983; Taylor *et al.*, 1983, 1986), and under some conditions ketogenesis (Taylor *et al.*, 1983; Kosugi *et al.*, 1983). Furthermore, the oxidation of octanoate is enhanced by hormones, but does not require carnitine acyltransferase to enter the mitochondria (Taylor *et al.*, 1983, 1986). In addition, in the starved state, where acetyl-CoA carboxylase is largely inhibited and carnitine acyltransferase I is relatively insensitive to inhibition by malonyl-CoA (Saggerson, 1982*b*; McGarry & Foster, 1983), glucagon can still stimulate fatty acid oxidation under some circumstances (Williamson *et al.*, 1969; Siess *et al.*, 1977, 1978*a*; cf. Christiansen, 1977; Brocks *et al.*, 1980). The same is true in biotin-deficient rats, where acetyl-CoA carboxylase would also be expected to be relatively inactive (Siess *et al.*, 1978*b*).

It has been suggested that a Ca^{2+}-dependent stimulation of isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase may be the locus of the intramitochondrial stimulation of fatty acid oxidation (Sugden & Watts, 1983; McCormack, 1985*a*,*b*; Taylor *et al.*, 1986). Although this mechanism may well explain increased flux through the citric acid cycle, it does not explain how the stimulation of β-oxidation is achieved. This occurs in the face of unchanged or slightly increased mitochondrial [ATP]/[ADP], [NADH]/[NAD^+] and [acetyl-CoA]/[CoA] ratios (Siess *et al.*, 1977, 1978*a*) and no measurable fall in proton motive force (Strzelecki *et al.*, 1984). We have provided evidence that under these conditions electron flow through the respiratory chain is stimulated at a point between ubiquinone and cytochrome *c* and that this is produced by an increase in intramitochondrial matrix volume (Halestrap, 1982; Armston *et al.*, 1982; Quinlan *et al.*, 1983; Quinlan & Halestrap, 1986). In the present paper we demonstrate that fatty acid oxidation in isolated mitochondria is particularly sensitive to changes in the matrix volume within the range of volumes expected *in vivo*. Furthermore, we show that it is electron flow from the electron-transferring flavoprotein (ETF) to ubiquinone that is the site of this effect of matrix volume.

Abbreviation used: ETF, electron-transferring flavoprotein.

EXPERIMENTAL

Materials

Mitochondria. Liver mitochondria were prepared as described previously (Halestrap, 1975) from female Wistar rats (250–300 g body wt.) allowed free access to food and water.

Chemicals. All chemicals, biochemicals and radiochemicals were from the sources quoted in Halestrap (1982) and Halestrap & Quinlan (1983). Ubiquinone-1 was generously given by Hoffman–LaRoche, Basel, Switzerland.

Methods

Mitochondrial respiration. The rate of oxygen uptake by liver mitochondria at various osmolarities was measured with a Clark-type oxygen electrode as described previously (Halestrap, 1982; Armston *et al.*, 1982). The buffer contained 10 mM-Mops, 7 mM-Tris, 2.5 mM-potassium phosphate, 2.5 mM-$MgCl_2$ and the required osmotic support. This was either 125 mM-KCl or, when variable osmolarities were used, 15 mM-KCl and sucrose to make up the osmolarity to the required value. The pH was 7.25 and the temperature 30 °C. Where ferricyanide rather than oxygen was to be used as terminal electron acceptor, the medium was supplemented with 2 mM-potassium ferricyanide and the rate of electron flow was measured spectrophotrometrically as described below. Further details are given in the legends to the Figures and Tables.

Measurement of mitochondrial matrix volume and light-scattering. Light-scattering was measured in a split-beam spectrophotometer as described previously (Halestrap *et al.*, 1986). For the measurement of mitochondrial volume, mitochondria (final concn. 4 mg of protein/ml) were mixed at 0 °C with buffer identical with that used in the oxygen-electrode experiments but containing 1 μCi of ^3H_2O/ml and 0.2 μCi of either [^{14}C]mannitol or [^{14}C]sucrose/ml. Five 1 ml samples were immediately centrifuged for the determination of the matrix volume as described previously (Armston *et al.*, 1982; Halestrap & Quinlan, 1983).

Measurement of ferricyanide and flavoprotein oxidation/reduction states. Ferricyanide reduction was measured in a double-beam spectrophotometer with the wavelength pair 440–490 nm and an absorption coefficient of 0.586 $mM^{-1} \cdot cm^{-1}$ (Osmundsen, 1981; Stoner, 1985). Flavoprotein oxidation and reduction were followed at 460–480 nm with a computerized spectrophotometer, with data averaging as described in Quinlan & Halestrap (1986). The mitochondrial protein concentration was about 5 mg/ml and the cuvettes were constantly stirred in an atmosphere of 100% O_2. Further details are given in the legends to Figs. 4 and 5.

RESULTS

Effects of osmolarity of the medium on rates of palmitoylcarnitine oxidation

The data of Fig. 1(*a*) show the effects of varying the osmolarity of the incubation medium on the rates of

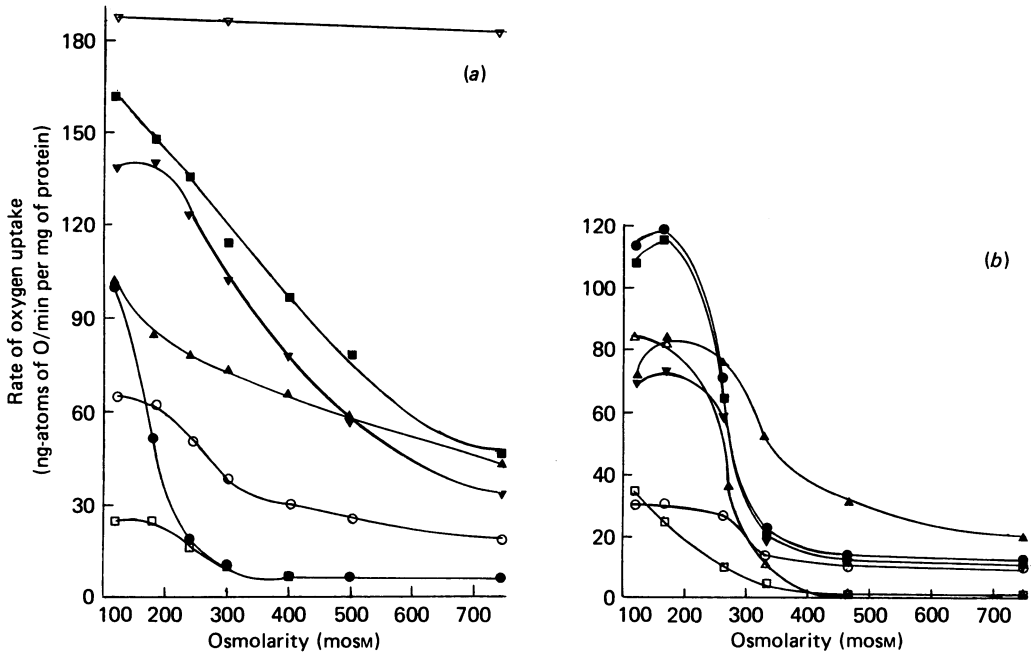

Fig. 1. Effects of varying the osmolarity on the oxidation of a variety of substrates by rat liver mitochondria

Rat liver mitochondria (1.5 mg of protein) were added to a sealed oxygen-electrode chamber containing buffer of the required osmolarity (see the Experimental section) and the relevant substrate. In (*a*) ADP was present at 0.5 mM and uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) at 0.2 μM. Substrates were 1 mM-malate in the absence (□) or presence of 5 mM-L-glutamate (▼) or 100 μM-palmitoyl-L-carnitine (●), 5 mM-succinate + 1 μg of rotenone/ml (■), 10 mM-L-proline (○), 10 mM-DL-β-hydroxybutyrate (▲) or 500 μM-duroquinol (▽). In (*b*) a separate mitochondrial preparation was used and no uncoupler was present. ADP was added in all cases but one (○). Substrates were 100 μM-palmitoyl-L-carnitine (●), 1 mM-octanoate (■), 2 mM-butyrate (▼), all in the presence of 1 mM-L-malate, 10 mM-DL-β-hydroxybutyrate (▲) and 100 μM-palmitoylcarnitine + either 2 mM-acetoacetate and 1 μg of rotenone/ml (□) or 1 mM-malonate (△). The rate of oxygen consumption was measured after 30 s equilibration. The experiment shown is typical of three such experiments.

uncoupled oxygen consumption in the presence of various substrates. The [KCl] of the medium was kept constant in these experiments and the osmolarity was varied with sucrose. We have previously published similar data for oxidation of succinate, glutamate + malate and ascorbate + *NNN'N'*-tetramethyl-*p*-phenylenediamine where KCl was used to vary the osmolarity. However, we have recently shown (A. P. Halestrap, unpublished work) that high [KCl] inhibits electron flow by causing cytochrome *c* displacement (see also Bookelman *et al.*, 1978). The present experiments show that the oxidation of duroquinol, like that of ascorbate, is insensitive to the osmotic strength. Data are shown only for 500 μM-duroquinol, but similar results were found at 50 μM. In contrast, all substrates entering the respiratory chain before ubiquinone show a marked sensitivity to the osmolarity. Data are shown in Fig. 1(*a*) for 10 mM-L-proline and 5 mM-succinate, which donate electrons to ubiquinone via specific flavoprotein-containing enzyme complexes (Jones, 1976; Johnson & Strecker, 1962; Erecinska, 1966), 5 mM-L-glutamate + 1 mM-L-malate and 10 mM-DL-β-hydroxybutyrate, which produce NADH and hence donate electrons to ubiquinone via NADH dehydrogenase, and 100 μM-palmitoyl-L-carnitine, which produces both NADH and reduced flavoprotein. The latter donates electrons to ubiquinone via a specific flavoprotein, ETF, and ETF dehydrogenase (Crane & Beinert, 1956; Ruzicka & Beinert, 1977). The oxidation of palmitoylcarnitine is particularly sensitive to changes in the osmotic strength. This was true whether malate was present, allowing maximal oxygen uptake through operation of both β-oxidation and the citric acid cycle, or when malonate was added to prevent citric acid cycle activity. Malate was added in most experiments to represent more closely the physiological situation. At osmolarities greater than 400 mosM the rate of uncoupled palmitoylcarnitine oxidation in the presence of 1 mM-malate was not significantly greater than the slow rate of oxidation of malate alone under the conditions used in Fig. 1. This inhibition of palmitoylcarnitine oxidation by high osmolarities was also observed in State 4 and under conditions where only electrons from the flavoprotein-linked oxidation were coupled to the respiratory chain (Fig. 1*b*). This was achieved by the addition of rotenone and 2 mM-acetoacetate to divert NADH to the production of β-hydroxybutyrate. Oxidation of both added octanoate and butyrate in the presence of ADP showed a sensitivity to osmolarity very similar to that displayed by palmitoylcarnitine (Fig. 1*b*). These data imply that it is not the entry of fatty acids into mitochondria via the carnitine/acylcarnitine antiporter that is the site of action of the increase in osmolarity, since both butyrate and octanoate enter the mitochondria by free diffusion and are activated in the matrix (Sugden & Williamson, 1981). Nor is it likely that it is the fatty-acyl-CoA dehydrogenase that is the site of inhibition, since different isoenzymes are used for the oxidation of short-, medium- and long-chain acyl-CoAs (Ikeda *et al.*, 1985).

Correlation of matrix volume with rates of fatty acid oxidation

Fig. 2 shows the relationship between the rates of State 4 and State 3 oxidation of palmitoylcarnitine and the intramitochondrial matrix volume measured under identical conditions. Volumes were measured with both [^{14}C]sucrose and [^{14}C]mannitol, to allow comparison

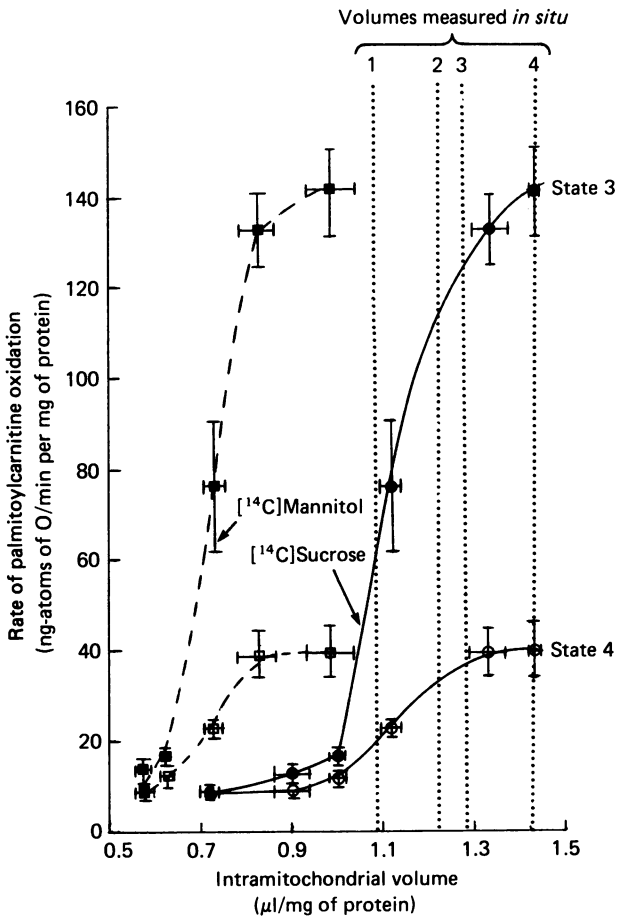

Fig. 2. Correlation of matrix volume with the rates of State-3 and State-4 palmitoylcarnitine oxidation

Mitochondria were incubated in media of different osmolarity and the rate of oxygen consumption and the matrix volume were measured. Data for the rates of palmitoyl-L-carnitine oxidation in State 3 (●, ■) and State 4 (○, □) are given as means ± S.E.M. (error bars) of five separate experiments performed as described in the legend to Fig. 1. The matrix volumes determined with either [^{14}C]mannitol (----; ■, □) or [^{14}C]sucrose (——; ●, ○) as described in the Experimental section, are given as means ± S.E.M. (error bars) for three separate experiments performed under conditions identical with the oxygen-electrode experiments but at 0 °C. The low temperature prevented swelling of the mitochondria occurring during the determination (see Fig. 3). The dotted vertical lines are the volumes of hepatocyte mitochondria *in situ* determined by Quinlan *et al.* (1983) in the absence (1) and presence of 0.1 μM-glucagon or 20 μM-phenylephrine (2), both hormones together or 25 nM-vasopressin (4) and 1 nM-valinomycin (3).

with data obtained previously for the volume of mitochondria *in situ* (Quinlan *et al.*, 1983). For reasons that we cannot explain, results obtained with [^{14}C]mannitol in recent experiments (see also Whipps & Halestrap, 1984) are greater than in earlier experiments (Halestrap & Quinlan, 1983) and make comparison with the data obtained *in situ* (Quinlan *et al.*, 1983) difficult. However, the [^{14}C]sucrose data are very similar to previous measurements, and the dotted lines on Fig. 2 show the values derived for the matrix volume *in situ*

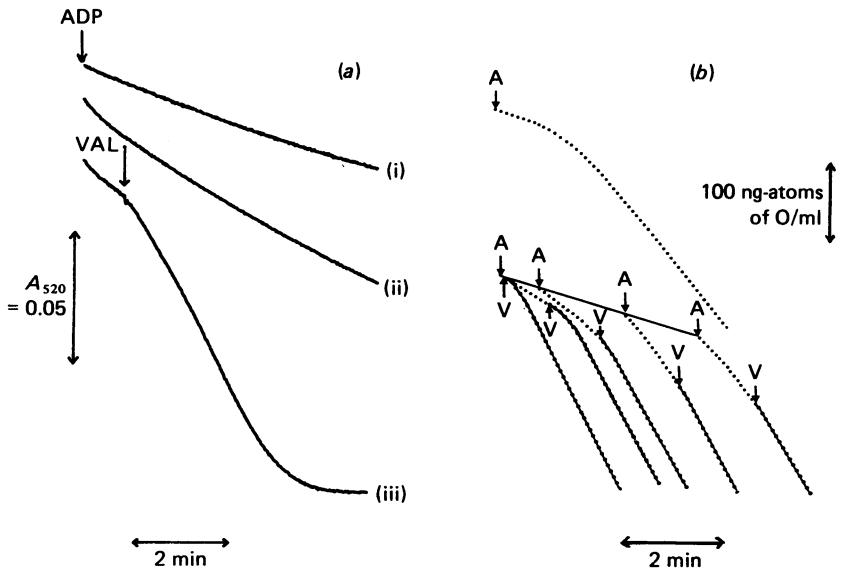

Fig. 3. Effects on palmitoylcarnitine oxidation of increasing mitochondria volume by incubating mitochondria in KCl medium

In (*a*) the swelling of mitochondria in KCl medium at 30 °C is shown as the decrease in light-scattering at 520 nM. The buffer contained 125 mM-KCl and other additions as described in the Experimental section. MgATP was present at 0.5 mM, L-malate at 1 mM and palmitoyl-L-carnitine at 100 μM. In (i) 1 mM-ADP was present from the start of the incubation, and in (iii) 1 nM-valinomycin (VAL) was added as indicated. Mitochondrial volumes were measured initially (parallel incubation at 0 °C) and at the end of each experiment by using [^{14}C]sucrose. Values (means $\pm$ S.E.M. for four determinations, expressed as μl/mg of protein) were 1.01 ± 0.033 (initial), 1.12 ± 0.030 (final), and 1.27 ± 0.046 (final + valinomycin). In (*b*) oxygen-electrode traces are shown from a parallel experiment where mitochondria were incubated in State 4 (——) for various times under the conditions used in (*a*) before addition of 1 mM-ADP (A, dotted line) or 1 nM-valinomycin (V, joined dots). The experiment shown is typical of three such experiments.

of control and hormone-stimulated hepatocytes. It is apparent that stimulation of fatty acid oxidation is occurring with increases of matrix volume within the physiological range.

We have shown that increases in volume caused by uptake of K^+ rather than induced by hypo-osmotic media also cause an increase in the rate of palmitoylcarnitine oxidation. Thus in Fig. 3 we show the decrease in light-scattering and increase in matrix volume that accompanies the energy-dependent swelling of mitochondria in KCl medium also produces an increased rate of palmitoylcarnitine oxidation. Indeed, in the presence of ADP the rate of oxygen consumption increases during incubation as the mitochondria swell. More rapid effects were seen on addition of 1 nM-valinomycin, which gave maximal rates of oxidation similar to those observed in the hypo-osmotic media.

The site of action of matrix volume on fatty-acylcarnitine oxidation

As outlined above, the site of action of volume on the oxidation of fatty-acylcarnitine is not the entry of the substrate into the mitochondria, nor is it likely to be the acyl-CoA dehydrogenases. Two possible sites of the effect are the reduction of ubiquinone by the ETF and its iron–sulphur-protein-containing dehydrogenase (Ruzicka & Beinert, 1977) or the oxidation of ubiquinol so produced. The latter possibility was investigated by studying the transfer of electrons to ferricyanide by using a double-beam spectrophotometer. The data of Fig. 4 show that the rate of electron flow from palmitoylcarnitine, glutamate + malate or succinate to ferricyanide at 500 mosM is less than that at 150 mosM either when electrons are transferred to ferricyanide through cytochrome *c* (the usual route) or when ubiquinone-1 and antimycin are present. These two additions allow electrons to flow directly from the ubiquinone pool to ferricyanide (Stoner, 1985). Again it is apparent that palmitoylcarnitine oxidation is particularly sensitive to the volume change. Rates of electron flow to oxygen and ferricyanide at both osmolarities and for all three substrates are given in Table 1.

The data of Fig. 4 indicate that the restriction of electron flow at low matrix volume is before the ubiquinone pool. In Fig. 5 data are presented to demonstrate that it is the transfer of electrons from ETF to ubiquinone that is affected. The reduction state of flavoproteins was measured by dual-beam spectrophotometry (460–480 nm). In Fig. 5(*a*) both hypo-osmotic (i) and hyperosmotic (ii) media were used. In the former case, addition of ADP and uncoupler to oxidize the endogenous substrates of the mitochondria caused oxidation of the flavoproteins concomitant with a small burst of oxygen consumption. This was assumed to represent the fully oxidized state. Addition of palmitoylcarnitine produced an immediate reduction of the flavoproteins and an increase in the rate of oxygen consumption. Antimycin was added finally to give the maximal reduction state of the flavoprotein. The calculated values of the percentage reduction of the flavoprotein in the presence of endogenous substrate and after addition of ADP, uncoupler and palmitoylcarnitine were about 12% and 50% respectively. In contrast, in the hyperosmotic medium addition of ADP and uncoupler produced neither an increase in the rate of oxygen uptake in the presence of endogenous substrates nor an

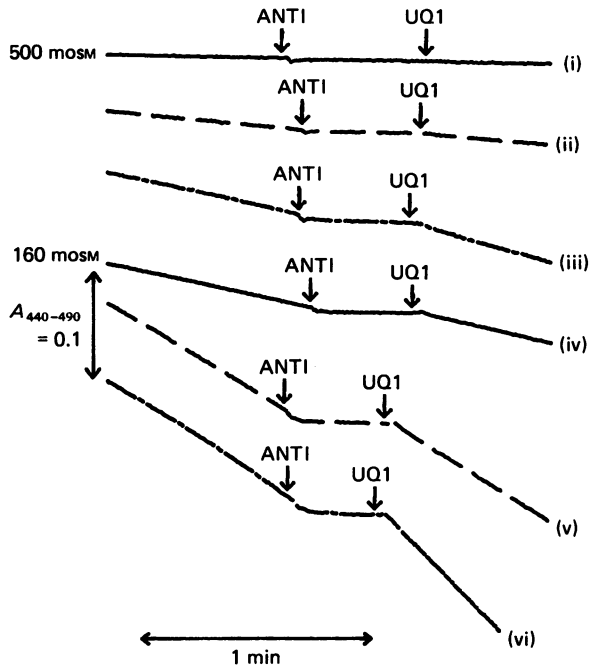

Fig. 4. Effects of mitochondrial volume on the uncoupled oxidation of various substrates with ferricyanide and ubiquinone-1 as electron acceptors

Incubations were carried out under conditions identical with those of Fig. 1 but in a double-beam spectrophotometer in the presence of 2 mM-potassium ferricyanide. Substrates were 100 μM-palmitoyl-L-carnitine + 1 mM-L-malate (i and iv), 5 mM-L-glutamate and 1 mM-L-malate (ii and v) and 5 mM-succinate + 1 μg of rotenone/ml (iii and vi). ADP was present at 0.5 mM and uncoupler at 0.2 μM. The media were either 500 mosM (i–iii) or 165 mosM (iv–vi). Where indicated 1 μg of antimycin/ml (ANTI) and 25 μM-ubiquinone-1 (UQ1) were added. Statistical analysis is given in Table 1.

oxidation of the flavoproteins. Addition of palmitoylcarnitine produced only slow rates of oxygen uptake and modest reduction of the flavoproteins. The addition of antimycin produced a small further reduction. Calculated values for the reduction state of the flavoproteins in the presence of endogenous substrates and after addition of ADP, uncoupler and palmitoylcarnitine were 70% and 80% respectively. These data show that in hypo-osmotic media faster electron flow is associated with more oxidized flavoproteins and imply that increasing the matrix volume stimulates electron flow from the flavoproteins to ubiquinone. The low rates of oxygen consumption seen in hyperosmotic media in the presence of endogenous substrate coupled to highly reduced flavoproteins imply that the endogenous substrates are fatty acids or their carnitine and CoA esters.

In Fig. 5(*b*) data are presented to demonstrate that the major component of the flavoprotein that is detected by measuring $A_{460-480}$ is ETF. The reduction state of flavoproteins was studied in KCl medium after various additions. Addition of glutamate + malate, rotenone or succinate had very little effect on the reduction of flavoproteins in comparison with palmitoylcarnitine. Only after antimcyin addition was there substantial reduction, and this would be expected to lead to reduction of all flavoproteins on the substrate side of the cytochrome bc_1 complex. Latipaa *et al.* (1986) have shown that high [NADH]/[NAD$^+$] ratios inhibit β-oxidation at the level of the 3-hydroxyacyl-CoA dehydrogenase. Trace (iii) of Fig. 5(*b*) demonstrates that high [NADH]/[NAD$^+$] ratios induced by the addition of rotenone cause oxidation of the flavoprotein, whereas addition of acetoacetate causes reduction. Thus inhibition of β-oxidation by increased [NADH]/[NAD$^+$] is also exerted at the level of the acyl-CoA dehydrogenase, and is consistent with the proposal that the enzymes of β-oxidation form a functional complex within the mitochondrial matrix (Stanley & Tubbs, 1975).

Table 1. Effects of mitochondrial volume on respiratory-chain activity in the presence of various substrates and electron acceptors

Mitochondria were incubated at 1.5 mg of protein/ml in either an oxygen electrode or a double-beam spectrophotometer as described in the Experimental section. All values are given as means ± S.E.M. for three experiments. The mitochondrial volumes measured in separate incubations using [^{14}C]sucrose and ^3H_2O were 1.40 ± 0.057 and 0.90 ± 0.20 μl/mg of protein (mean ± S.E.M.) for 165 and 500 mosM incubation buffers respectively. Statistical significance of the difference between values at the two osmolarities was calculated by Student's *t* test: $^*P < 0.01$.

Substrate	Electron acceptor	Rate of acceptor reduction at 165 mosM (nmol/min per mg of protein)	Rate at 500 mosM (% of that at 165 mosM)
Glutamate + malate	Oxygen	135 ± 15.8	48.6 ± 2.5*
	Ferricyanide	137 ± 32.0	21.6 ± 2.7*
	Ubiquinone-1	143 ± 31.5	18.0 ± 3.2*
Palmitoylcarnitine + malate	Oxygen	116 ± 18.3	9.6 ± 1.3*
	Fericyanide	69 ± 8.0	8.4 ± 2.8*
	Ubiquinone-1	80 ± 9.7	6.3 ± 3.0*
Palmitoylcarnitine + rotenone + acetoacetate	Oxygen	27 ± 3.7	10.7 ± 2.4*
Succinate + rotenone	Oxygen	156 ± 16.9	65.6 ± 3.5*
	Ferricyanide	162 ± 30.8	35.2 ± 3.2*

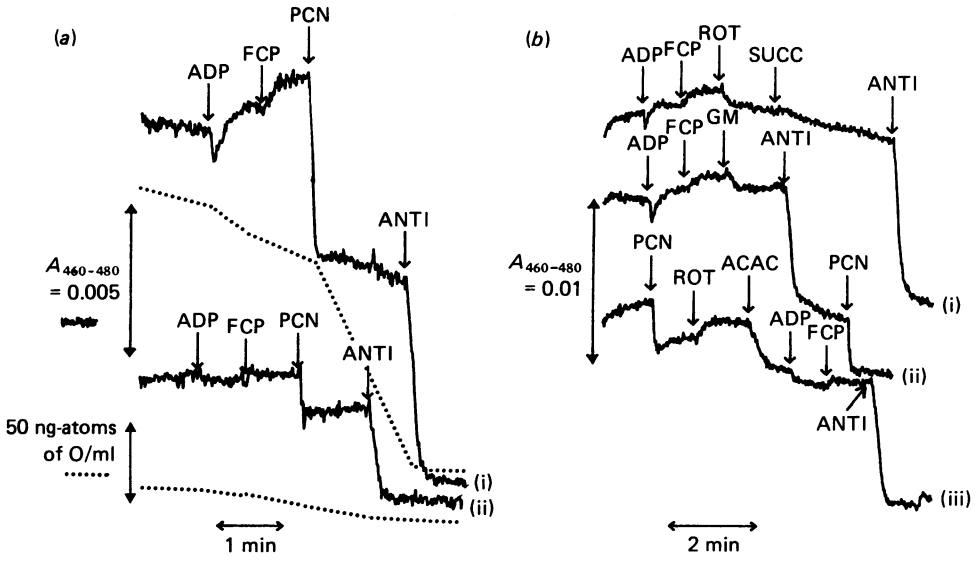

Fig. 5. Reduction state of flavoprotein with various substrates and at two osmolarities

Mitochondria were incubated at 5 mg of protein/ml and 30 °C in a double-beam spectrophotometer, and the $A_{460-480}$ was measured as described in the Experimental section. In (*a*) osmolarities were adjusted to 165 (i) and 500 (ii) mosM with sucrose, and in (*b*) normal KCl medium was used. Additions were made as indicated: 0.5 mM-ADP (ADP), 0.2 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCP), 100 μM-palmitoyl-L-carnitine (PCN), 1 μg of antimycin (ANTI) or rotenone (ROT)/ml, 5 mM-succinate (SUCC), 5 mM-L-glutamate + 1 mM-L-malate (GM) and 2 mM-acetoacetate (ACAC). In (*a*) oxygen-electrode traces (dotted lines) are shown from parallel incubations at the two osmolarities.

DISCUSSION

The data presented in Figs. 1–3 of this paper show that β-oxidation of palmitoylcarnitine is very sensitive to changes in the matrix volume between 1.0 and 1.5 μl/mg of protein, whether the volume is changed by electrogenic uptake of K^+ or by varying the osmotic support. This conclusion has also been reached by Otto & Ontko (1982), whose results complement those presented here. However, those authors did not provide detailed matrix-volume measurements, and were unable to define as precisely the locus of the effect of volume. In agreement with Otto & Ontko (1982), we find that the effect is independent of the chain length of fatty acid used and is distal to the transport of the fatty-acyl-CoA into the mitochondria, since the carnitine-independent oxidations of butyrate and octanoate are also volume-sensitive. That is not to say that volume may not also affect the entry of fatty acids into the mitochondria by the carnitine-dependent pathway. Saggerson (1982*a*) has shown that carnitine acyltransferase activity is enhanced by the presence of KCl in the incubation medium, and such conditions are known to cause swelling of mitochondria (Halestrap *et al.*, 1986). However, the effect is seen in sonicated mitochondria and is reversed at high [K^+], which argues against the effect being volume-mediated. The data of Figs. 4 and 5 suggest that the major locus of the effect of matrix volume on β-oxidation is between ETF and the ubiquinone pool. This flavoprotein appears to be held reduced under conditions of low matrix volume, and this is not prevented by addition of exogenous ubiquinone-1 or ferricyanide. This would imply that either electron transfer from ETF to the iron–sulphur protein of its dehydrogenase (Ruzicka & Beinert, 1977) or the access of ubiquinone to this iron–sulphur centre is limited under conditions of matrix condensation. How this is achieved can only be conjectured, but it would seem likely to involve a conformational change of the mitochondrial inner membrane. We have shown previously that oxidation of all substrates entering the respiratory chain before ubiquinone can be stimulated to some degree by increasing the matrix volume (Armston *et al.*, 1982; Halestrap, 1982). This effect is still seen when electrons by-pass the cytochrome bc_1 complex and are transferred to ferricyanide via ubiquinone-1 (Fig. 4). Thus it would seem quite likely that the transfer of electrons from the flavoproteins of NADH dehydrogenase and succinate dehydrogenase via specific iron–sulphur proteins to the ubiquinone pool is also the step affected by matrix volume when succinate, proline and NADH are oxidized.

Physiological significance of volume-mediated stimulation of fatty acid and proline oxidation

Our measurements of the mitochondrial matrix volume in isolated hepatocytes (Quinlan *et al.*, 1983) give a basal value of about 1.1 μl/mg of mitochondrial protein, rising by 20–40% with hormones as shown by the broken line in Fig. 2. This is the same range over which β-oxidation may be regulated by the matrix volume. As outlined in the Introduction, the conventional view of the regulation of β-oxidation is by malonyl-CoA-dependent control of carnitine acyltransferase I, although there is substantial evidence to support an intramitochondrial locus also. Some workers have suggested that Ca^{2+}-dependent activation of the mitochondrial dehydrogenases may be responsible for this (Sugden & Watts, 1983; McCormack, 1985*a*,*b*; Taylor *et al.*, 1986), and there is strong evidence for this occurring (see Denton & McCormack, 1985; Quinlan & Halestrap, 1986). However, stimulation of fatty acid oxidation by stimulating the activity of isocitrate dehydrogenase and

2-oxoglutarate dehydrogenase requires some link between the citric acid cycle and β-oxidation. No such link is known. Latipaa *et al.* (1986) have shown that increased [NADH]/[NAD$^+$] ratios can inhibit β-oxidation at the level of 3-hydroxyacyl-CoA dehydrogenase, whereas acetyl-CoA has no major feedback-inhibitory effect. Both glucagon and Ca^{2+}-mobilizing hormones cause an increase in [NADH]/[NAD$^+$] (see Quinlan & Halestrap, 1986) and an increase or no significant change in acetyl-CoA concentrations as measured either directly (Siess *et al.*, 1977; Chisholm *et al.*, 1983) or indirectly by total ketone-body production (see the Introduction). Thus their effects on fatty acid oxidation cannot be exerted by either of these means. However, a Ca^{2+}-mediated increase in matrix volume (Halestrap *et al.*, 1986) coincident with an increase in citric acid-cycle activity could provide the necessary co-ordinate activation of β-oxidation and the cycle activity. Some evidence for this linkage is provided by the observation that the increase in NAD(P)H fluorescence induced in hepatocytes by gluconeogenic hormones is greatly inhibited by the fatty acid oxidation inhibitor pent-4-enoate (Quinlan & Halestrap, 1986). It is of interest that Otto & Ontko (1982) also provided evidence that the stimulation of fatty acid oxidation that they observed after adding Ca^{2+} to isolated mitochondria was due to an increase in matrix volume rather than changes in [NADH]/[NAD$^+$] or [ATP]/[ADP] ratios. In view of the strong stimulatory effect of fatty acid oxidation on gluconeogenesis and its prevention by inhibitors of fatty acid oxidation, the ability of hormones to increase matrix volume may be central to the mechanism by which they stimulate gluconeogenesis (Quinlan *et al.*, 1983; Quinlan & Halestrap, 1986). The stimulation of proline oxidation and conversion into glucose observed both in isolated liver cells (Staddon & McGivan, 1984) and in the perfused liver (Haussinger *et al.*, 1982; Haussinger & Sies, 1984) may also be explained by a Ca^{2+}-dependent increase in the matrix volume and stimulation of the citric acid-cycle dehydrogenases.

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